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Human breast cancer cell lines and invasive tumors frequently have reduced tropomyosin (TM) expression. We wish to identify the normal function of the TM isoforms expressed in breast epithelial cells and detemine if altered TM expression has prognostic value in human breast cancer. The first step is to determine which TM isoforms most frequently exhibit altered expression in human breast cancers, compared to normal, mortal human mammary epithelial cells (HMEC). Using Northern and Western blot analysis to characterize TM expression in an *in vitro* cell culture model of human breast cancer we determined that TM1 RNA is reduced in the breast cancer cell lines SKBR3, MCF7, T47D and MDA-MB-435 while in MDA-MB-231 cells, TM1 RNA is increased, relative to normal, mortal HMEC, correlating with reduced TM1 protein. TM4 RNA is increased in the benign, immortalized breast cell lines HBL100, 184A1N4 and in the cancer cell lines 184A1N4TH and SKBR3, but shows no significant differences in abundance in MDA-MB-231, MCF7, T47D and MDA-MB-435 breast cancer cells. TM4 protein is reduced in the latter four cell lines. Immunohistochemistry performed on human breast tumor samples reveals decreased HMW TM (TM1) expression in sixty per cent of infiltrating ductal carcinomas examined thus far.

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INTRODUCTION:

The molecular changes driving tumorigenesis in the breast are poorly understood. Many of the molecular alterations documented do not occur consistently from tumor to tumor. There are only a few which occur in a larger percentage of tumors. For example, point mutations in the tumor suppressor gene *TP53* have been among the most consistently identified molecular defects, albeit with a variable frequency (40 - 70%; 1). Indeed, many molecular changes have been found in the cells from a variety of tumors (2). There is however, a diversity with respect to the changes which can occur in a particular type of tumor (e.g. breast tumors, reviewed in 3). Thus, although all breast tumors undoubtedly have defects at the molecular level, the frequency of any one particular defect is usually low (e.g. 5-20%; 3, 4).

Among the changes consistently identified in a variety of transformed cell types, including breast epithelial cells, are changes in expression of cytoskeletal proteins (e.g. actin, gelsolin, and tropomyosin; 5-7). These proteins play a role in several fundamental cell processes including maintaining cell shape and regulating cell motility and normal proliferative growth (8-11). It is therefore not surprising that transformed cells are often altered in morphology and more motile than their normal counterparts (12).

Tropomyosins (TMs) have altered expression in many transformed cell types (5, 12-15). TMs are actin binding proteins which help to regulate the Ca^{2+} sensitivity of contraction in muscle cells (16) and may regulate cell motility and intracellular vesicular transport among other processes, in non-muscle cells (17). Their expression is normally subject to complex cell-type-specific regulation involving multiple promoter usage and alternative splicing (16). Rearrangement of the α-tropomyosin gene is responsible for directing expression of the trk oncogene fusion (18). Over-expression of some TM isoforms inhibits transformation and

restored expression causes reversion of the transformed phenotype (19, 20). These observations strongly suggest a tumor suppressor function for tropomyosin genes.

Bhattacharya et al., (13) used two dimensional polyacrylamide gel electrophoresis (2D-PAGE) of immunoprecipitated TMs to demonstrate consistent loss of high molecular weight (HMW) tropomyosin isoform expression in tumorigenic mammary epithelial cells compared to primary human mammary epithelial cells (HMEC) and immortalized HMEC. Quantitative changes were also found in TM isoform expression reminiscent of "isoform switching" seen in transformed fibroblasts and thyroid epithelial cells (21,22). The authors also identified two possible epithelial cell specific isoforms of TM (38kD and 32kD). Franzen et al. (15), have examined TM isoform expression by 2D PAGE in breast cancer cell lines, in cells from non-cancerous breast lesions and tumor cells purified from breast carcinomas. This study demonstrated the reduced expression of HMW TM isoforms (TMs 1, 2 and 3) in breast tumor cell lines and in infiltrating ductal carcinomas relative to non-cancerous breast lesions. Importantly, additional analysis suggested that TM1 expression was increased in the cells from node positive tumors relative to node negative tumors. The number of lesions examined, however, was relatively small. The potential association of TM1 (and perhaps other TM isoforms) expression with nodal status is a significant observation which warrants follow up with analysis of a large series of breast cancer samples. Results in this laboratory have also demonstrated a loss or reduction of HMW TM isoform expression in a number of breast cancer cell lines using indirect immunofluorescence or western blotting and in infiltrating ductal carcinomas employing immunohistochemistry on frozen sections.

These data demonstrate the relevance of assessing TM proteins as markers of tumor progression. Screening of large numbers of breast tumors is required to establish the true

incidence of altered TM expression and its value as a prognostic or progression marker. Additionally, the mechanisms responsible for aberrant TM expression must be determined. This knowledge is needed in order to understand both the normal function(s) of TMs and how alterations in TM expression contribute to the growth and spread of breast cancer cells. Understanding these mechanisms will help to determine if TM proteins represent new targets for therapeutic intervention and is the minimum needed to design rational approaches to such therapy.

EXPERIMENTAL METHODS, ASSUMPTIONS AND PROCEDURES:

To assess TM expression in breast cell lines both TM RNA and protein levels were examined. Comparison of TM expression was done using an *in vitro* cell culture model consisting of normal, mortal (HMEC), benign, immortalized (HBL100, MCF10A and 184A1N4) and carcinomatous (184A1N4TH, SKBR3, MDA-MB-231, MDA-MB-435, MCF7 and T47D) breast epithelial cells (23). This will allow for establishment of the normal expression pattern and for the determination of differences which can occur during breast tumorigenesis. Normal breast epithelial cells were grown in MCDB 170 medium, HEPES buffered and supplemented with hydrocortisone, insulin, EGF and bovine pituitary extract (Clonetics). The benign, immortal breast cell lines HBL100, MCF10A and 184A1N4 and the breast cancer cell lines 184A1N4TH, SKBR3, MDA-MB-231, MDA-MB-435, MCF7 and T47D were propagated in DMEM/F12 or DMEM supplemented with 5% FBS as reported in (24). All cells were incubated at 37°C in a 5% CO_2 atmosphere until harvested for isolation of nucleic acids or protein as described below.

Steady state TM RNA levels were determined using Northern blot analysis of RNA from the normal, benign immortal and carcinomatous breast cell *in vitro* model (Fig. 1A) and RNA from carcinoma cell lines treated with trichostatin A (TSA, a histone deacetylase inhibitor; 25), 5-azacytidine (5aza, a DNA methyltransferase inhibitor 26) or a combination of the two agents (Fig. 1B and 1C). TSA, 5aza and 6aza (an inactive analog of 5aza) were purchased from Sigma Corp. (St. Louis, MO). The breast cancer lines chosen, were MDA-MB-231, MCF7 and T47D. These lines are used to represent estrogen-independent (MDA-MB-231) and estrogen-dependent (MCF7 and T47D) breast cancer growth (23). Moreover, they demonstrate the two extremes of TM1 expression (see discussion). For treatment with TSA, breast cancer cell lines were grown and plated into culture dishes, switched to medium containing 100ng/ml TSA, incubated for the indicated times, and then harvested for isolation of RNA. For 5aza treatment, breast cancer cells grown as above were trypsinized and seeded into $10cm^2$ tissue culture dishes containing medium alone or with either 5 uM 6aza or 5 uM 5aza. Cells were incubated for 72 h, changed to fresh medium, incubated for an additional 24 h (96 h total with either 6aza or 5aza) and harvested for isolation of cytoplasmic RNA as in (27). For combining 5aza and TSA, cultures were treated with 5aza the same as above except that cells were given fresh medium with 5μM 5aza after 60 h instead of 72 h. Cultures treated with 5aza plus TSA were changed to fresh medium containing 5μM 5aza plus 100ng/ml TSA after 60 h and incubated further for the indicated periods of time before being harvested for the isolation of RNA. Analysis of TM levels using these extracts will indicate if there may be epigenetic effects on TM isoform expression in human breast cancer cells. Total or cytoplasmic RNA was isolated from cells using the guanidine isothiocyanate method (28), fractionated on a 1.0% agarose/formaldehyde gel, transferred to a nylon

membrane (Zetabind; CUNO Inc., Meriden CT) and sequentially hybridized first with ^{32}P-labeled isoform-specific TM DNA probe (either TM1-, 3-, 4- or 5-specific), followed by a fragment of 18S rDNA, after stripping of the first probe. Hybridization was at 65 °C using the method in (29). Hybridization signals were quantified using a Molecular Dynamics laser scanning densitometer and Imagequant software. Signals obtained from TM probes were corrected for loading by normalization to those obtained for 18S RNA.

In parallel experiments, cell cultures were harvested at the indicated times for isolation of protein as in (24). Treated or untreated cells were collected in the presence of protease inhibitors. Total cellular proteins were solubilized in Laemmli buffer and Western blot analysis was performed as in (24). Equal amounts of protein (as determined by densitometry of Coomassie blue stained parallel gels) were loaded in each lane. Protein transfer was monitored by India ink staining after immunoblotting. The antibodies used were mAb TM311 (SIGMA), which is HMW specific on western analysis, but on 2D PAGE it detects six immunoreactive spots, including TMs 4 and 5 (30), mAb TM228 (SIGMA) and mAb CG1 (31). Some individual blots were washed in PBS plus 0.1% Triton-X 100, reblocked and immunostained with a monoclonal β-actin antibody (clone C4; ICN, Aurora, OH) to determine total cellular actin levels. Western blot analysis was used to assess TM protein expression in the same human breast epithelial cells in which TM RNA expression has been assessed (Fig's. 2 and 3). Chemiluminescence signals were quantitated as for RNA with correction for loading differences (on TSA and TSA plus 5aza blots) obtained by normalization to β-actin signals. Immunohistochemistry (ABC peroxidase method) was utilized to detect TM isoform expression in frozen tumor sections (Table 1) and in sections cut from formalin fixed, paraffin embedded

tumors (Fig. 4; Table 2). The IH procedure was performed on frozen sections adhered to glass coverslips as described in (24) and on paraffin sections using the Ventana 320 automated immunostainer after microwave antigen retrieval. Slides with paraffin sections were counterstained with hematoxylin after IH.

RESULTS AND DISCUSSION:

Individual tropomyosin isoform RNA expression in smooth muscle cells and fibroblasts can be regulated by a number of factors, including expression of viral oncoproteins, serum stimulation, agents which increase cyclic AMP levels, exposure to inflammatory cytokines, or modulation of actin levels (5, 14, 30, 32-34). Changes in TM RNA expression typically coincide with the phenotypic alterations of these cells in response to a specific treatment (30, 32). This situation is analogous to the changes in phenotype of breast cancer cells relative to normal breast epithelial cells.

In human cells there are four TM genes (α, β, γ and δ; 17) from which up to twelve TM protein isoforms are derived by way of cell-type specific splicing and/or alternative promoter usage (17). Human fibroblasts express RNAs derived from the TM-α (a 2.0 kb transcript encoding TMs 2, 3 and sm- α, and a 1.9 kb transcript encoding TM5a & 5b), TM-β (a 1.1 kb transcript encoding TM1), TM-γ (a 2.5 kb transcript encoding TM5, 2.5kb) and TM-δ (a 3.0 kb transcript encoding TM4) TM genes (17). Breast epithelial cells appear to express seven TM protein isoforms: p39, p38, p36, p35, p33a, p33b, p32 (13), but it has not been definitively shown which gene products these proteins represent. TM1 RNA (transcribed from the TM-β gene) is expressed in normal, mortal 184 breast cells and its levels are decreased in breast cancer cell lines (13). RNA expression from other TM genes in human breast cells has not been

examined. Additionally, little is known concerning the regulation of TM RNA expression in normal human breast cells.

To begin characterization of TM isoform RNA expression, Northern analysis was performed on RNA from the breast cell lines (Fig. 1A). Quantification of the signals obtained revealed a greater than 95% reduction in the level of TM1 RNA in SKBR3, MCF7 and T47D breast carcinoma cells. MDA-MB-231 breast cancer cells had a slight elevation (< 2-fold) in TM1 RNA. These results are nearly identical to those obtained by Bhattacharya et al., (13), using a full length TM1 cDNA (TMe1). RNA encoding the LMW isoform TM5 does not differ substantially in any of the breast cells while RNA encoding the LMW isoform TM4 is elevated 3.5- and 3.8-fold in benign, immortalized HBL100 and 184A1N4 cells, respectively, and 3.5- and 5-fold in 184A1N4TH and SKBR3, respectively. Unfortunately, I have been unable to obtain the TM2-specific DNA probe I have requested and, a TM3 probe purchased from the ATCC detects a transcript of less than 1 kb in size (TM3 mRNA is 2.0 kb). In addition, there currently is no TM2- or TM3-specific antiserum available. The mAb CGβ3 (31) detects both TM2 and TM3 proteins. Thus TM2 and TM3 expression must be distinguished indirectly, employing differential detection of TM2 and/or TM3 RNA (both 2 kb in length) with specific DNA probes and TM2/TM3 protein using CGB3. A TM2 probe will be derived by PCR amplification, from the human α-TM gene, of DNA encoding amino acids 189-213, which are common to TM2, TM5a and TMsmα. Recently, I have received the full length human TM3 cDNA (pGEMhTM3; 36) from which I will derive the TM3-specific probe fragment, encoding aa 189-213, which are common to TM3 and TM5b (35). These methods require the absence of either TM2 or TM3 RNA to infer which protein mAb CGβ3 detects. Pep3-43 mAb raised against TM5a and 5b (36) will be used to determine if the TM2 or the TM3 DNA probe also

detected co-migrating TM5a or TM5b RNA (1.9 kb), respectively. Separation of the RNA samples using ≥2.0% agarose gel electrophoresis may also allow for resolution of these transcripts. Because of these possibilities, however, there is a chance that I may not be able to distinguish between TM2 and TM3 or TM5a and TM5b expression in these analyses.

Northern analysis performed on RNA from untreated, TSA-, 5aza- or TSA plus 5aza-treated MDA-MB-231, MCF7 or T47D breast cancer cells shows possible epigenetic regulation of TM1 RNA levels in MDA-MB-231 cells. Both 5aza and TSA down-regulate TM1 RNA expression (>65% and>99% maximum reductions, respectively; Fig. 1B and 1C). 5aza plus TSA treatment resulted in an approximately 95% maximum reduction in TM1 RNA (Fig. 1C). TM1 expression in MCF7 and T47D cells was unaffected by any of the treatments (data not shown).

As mentioned above, human breast epithelial cells express seven TM isoforms, identified by 2D PAGE analysis of immunoprecipitated proteins (13). These may be the same proteins identified in fibroblasts or they may represent some of the same isoforms plus some epithelial cell specific isoforms (13). This situation contrasts that of normal colon or jejunal epithelial cells, where TM4 and TM5 are the predominant isoforms detectable by immunoblot analysis using isoform-specific antisera (37). Thus breast epithelial cells seem to differ in TM protein profile from intestinal epithelial cells. Additionally, epithelial cells from breast tumors have reduced TM 1, 2 and 3 isoforms with TM1 expression seemingly higher in node positive tumors relative to node negative tumors (15).

To begin to address the question of TM protein isoform expression in breast epithelial cells, Western blot analysis of proteins isolated from human breast cell lines was performed using mAb TM311 to examine HMW TM expression. This experiment confirmed our preliminary results reported earlier (data not shown) which showed differential detection of two distinct

immunoreactive bands in SKBR3 and MDA-MB-231 extracts relative to normal HMEC (both bands present), and the absence of both of those bands in MCF7 and T47D extracts. The absence of the upper isoforms correlates with loss of the TM1 RNA (with the exception of MDA-MB-231). In addition, extracts from MDA-MB-435 breast cancer cells also show a complete lack of immunoreactivity with mAb TM311 (Fig. 2). It is known that the highest molecular weight TM isoform expressed in fibroblasts is TM1 (39/40kD; 30, 38) and it has been suggested that the human breast epithelial cell p39 is TM1 (13). Our preliminary results initially suggested a higher M_r (approximately 45 kD) for the upper band identified by TM311 on immunoblot analysis of human breast epithelial cell proteins compared to the M_r previously published . Subsequent experiments (Fig. 2A) confirmed that the upper band co-migrates with the 45kD standard we use in our laboratory (Kaleidoscope prestained MW standards, BIORAD). The relative mobility of the prestained standards has been calibrated by the company and differs from lot to lot. Thus, they can only be relied upon for molecular mass estimates. Moreover, the upper band detected by TM311 migrates just below the 43kD actin band (data not shown). Taken into account, these observations suggest that the mobilities of the bands detected using anti-HMW tropomyosin antibodies is in relative agreement with those of previous studies (approximately 40kD; 13, 30, 38). However, more precise determinations need to be made to confirm this. Nonetheless, the presence of the upper band in MDA-MB-231 cells (Fig.2A) contrasts with the identification of TM1 as the isoform whose expression is lost in these cells (13). This is confirmed in Fig. 2B using the TM1-specific mAb CG1 (37). CG1 detects two isoforms in mouse 3T3-L1 fibroblasts and in HMEC, 184A1N4 and MCF10A cells (Fig. 2B, lanes 1-4). It also detects two isoforms in MDA-MB-231 cells with the upper band being very in similar moblility to the band detected by TM311 (Fig. 2B, lane 6, compare with Fig. 2A, lane 6). These observations are consistent with

the presence of TM1 RNA in MDA-MB-231 cells (detected with an isoform-specific DNA probe). In all cells in which it is detected, the upper band recognized by TM311 has a mobility very similar to that of the upper TM1 isoform identified by CG1 (compare Fig. 2A and 2B), lending further support to its identification as TM1. The faster mobility TM1 isoform identified in Fig. 2B seems to have a slightly faster mobility in mouse fibroblasts than it does in human breast cells (compare lane 1 to lanes 2-5, 6 and 9). Other differences detected by mAb CG1 are an absence of the faster TM1 isoform in SKBR3 cells and the presence of it in MDA-MB-435 cells. The significance of these differences is unclear at present. Fig. 2C shows Western blot analysis from the most of the same cells as in Fig. 2B (lane 3 is HBL100 instead of 184A1N4) using mAb LC24, which is specific for the LMW TM isoform TM4 (37). 3T3-L1 fibroblasts lack any TM4 immunoreactive proteins. Surprisingly, the antibody detects two TM4-related isoforms in HMEC and in each of the benign breast cell lines. As was seen for TM1 isoforms, breast cancer cells also show changes in TM4 isoform composition. MDA-MB-231 cells express both isoforms (at a different ratio than HMEC) while MDA-MB-435 cells expresses only the upper form. SKBR3, MCF7 and T47D cells lack both of these TM4 variants. This contrasts with TM4 RNA expression which does not show any significant reduction in the breast cancer cell lines.

The CG1 and LC24 antibodies have been shown to be specific for TM1 and TM4 without any cross-reactivity to other TM isoforms (37). It could thus be argued that the lower bands detected by CG1 and LC24 are degradation products of TM1 and TM4, respectively. Generally, however, immunodetection of actin and India ink staining both suggest that protein degradation does not occur to any great extent in our extracts. Nevertheless, although we include protease inhibitors during the cell harvesting and protein extraction processes, it is still possible that there is a TM-

specific protease activity which is not inhibited. CG1 detects a single band in human intestinal epithelial smooth muscle cells (37) and, it detects two bands, similar in mobility to those detected here, in chicken embryo fibroblasts (31). The specific differences in TM1 and TM4 isoforms detected between cell lines, however, argues against their resulting from TM-specific protease activity and suggests that they are, in fact bonafide TM1- and TM4-related isoforms which may be specific to human breast epithelial cells.

Western blot analysis of protein extracts derived from untreated, 5aza-, TSA-, or TSA plus 5aza-treated MDA-MB-231, MCF7 or T47D cells using mAb TM311 reveals down-regulation of an the upper TM311 immunoreactive band in MDA-MB-231cells (>27%, >60% and >80%, respectively; Fig. 3). This correlates with 5aza- and TSA-induced down-regulation of TM1 RNA and further supports the possiblility of epigenetic regulation of TM1 expression in these cells. This will be confirmed using mAb CG1.

Immunohistochemistry (IH) was performed on frozen sections of human breast tumors. Analysis of these tumors is still in its preliminary stages, but we have analyzed several additional samples and these results are summarized in Table 1. Examples of results obtained by IH analysis performed with mAb TM311on sections from paraffin embedded tumors are shown in Fig. 4. Panel A reveals that the antibody stained myoepithelial cells of normal ductal structures very intensely (long arrows) while luminal cells of normal breast tissue were less intensely stained (moderate to strong staining; short arrows). Stromal cells (thin arrows) were always strongly positive with mAb TM311 and, along with the residual normal tissue components, served as an internal positive control in tumor sections. Panel B shows TM-positive tumor cells from an infiltrating ductal carcinoma. Most of the invasive tumor cells show moderate to strong staining with TM311 (long arrows), with some cells staining more weakly (short arrows). Panels

C and D of show examples of TM-negative infiltrating ductal carcinomas. Virtually all of the invasive tumor cells are negative for TM immunoreactivity (long arrows). Note the presence of positively staining stromal (thin arrows, panels C & D), residual myoepithelial cells (short arrows, panels C & D) and normal ductal components (thick arrows, panel D). A summary of the IH results with paraffin embedded tumor sections is given in Table 2. Although preliminary, these results are consistent with those obtained on frozen breast tumor sections. Combined, these data show that 59.9% of tumors examined to date show reduced or absent TM expression.

In addition to the TM311, TM228, CG1 and LC24 antisera, several other TM isoform-specific mAbs are now in hand and ready for use in both Western and IH analysis. These are: 1) mAb CGβ6 which detects both TM2 and TM3, 2) mAb LC1 which is TM5-specific, 3) Pep3-43 which is specific for TM5a and TM5b . These experiments are currently underway.

RECOMMENDATIONS RELATED TO THE STATEMENT OF WORK (SOW):

Task number one, relating to the preparation of histological specimens, DNA/RNA probe constructs and titration of antisera, is nearly complete. The preparation of the alternative TM2/TM5b and TM3/TM5a DNA probes should be completed soon. In addition, I now have all of the antisera needed to identify specific TM isoform expression in human breast cells. Thus tasks two and three, relating to the characterization of TM expression in normal and tumorigeneic breast epithelial cell lines and in paraffin embedded tumors should proceed without any delays and will also be completed shortly. As shown in figures two and three, however, interpretation of the data may reveal a fairly complex pattern of Tm expression. Progress should be made on task four after examination of additional tumor sections, as the current number analyzed is still too low for statistical reliability. Additionally, progress has been made on specific aim two, relating to regulation of TM RNA and protein expression.

CONCLUSIONS

We have shown that TM1 is down-regulated at the level of both RNA and protein in some breast cancer cells. In others, TM1 expression may be subject to epigenetic regulation. We also have shown TM4 proteins are absent in a subset of the breast cancer cells which lack TM1 expression. Surprisingly, normal human breast epithelial cells express two isoforms each of TM1 and TM4. Additionally, it seems that there is differential regulation of TM4 RNA and protein in human breast cancer cells. It will be interesting to see how many total TM proteins are expressed in breast cells. Clearly, the pattern of TM protein expression is going to be quite complex and there may be several distinct alterations of the TM expression profile which can contribute to the phenotypic characteristics of human breast cancer cells.

In other studies in this laboratory, we have shown epigenetic regulation of the actin-binding protein gelsolin in the same breast cancer cells examined in this study which show possible epigenetic regulation of TM1 expression. This is important in light of the interactions which can occur between gelsolin and tropomyosin (39).

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Table 1. Summary of frozen section staining reactions with mAb TM311.

TISSUE ID	STAINING PATTERN	CATEGORY
N[1]	Luminal cells = 3+ myoepithelial cells = 4+	P
54T	Invasive tumor cells = +/-	R
67T	Invasive tumor cells = 3-4+	P
72T	Invasive tumor cells = 0	N
75T	Invasive tumor cells = 1-4+	P
77T	Invasive tumor cells = 3-4+	P
81T	Invasive tumor cells = 0	N
83T	Invasive tumor cells = 0	N
87T	Invasive tumor cells = 1-2+	R
89T	Invasive tumor cells = 2-3+	P
91T	Invasive tumor cells = 0 Intraductal cells = 3+	N
93T	Invasive tumor cells = 0	N
95T	Invasive tumor cells = 2+	R
Totals:	25% Positive; 75% Reduced or Negative	

[1] N = Normal, T = Tumor

Staining was performed on acetone-fixed frozen sections mounted on glass coverslips. Antibody was detected by the ABC Peroxidase method Vectastain reagents (Vector Laboratories, Inc., Burlingame, CA). See Table 2 for scoring criteria.

Table 2. Summary of immunohistochemistry on paraffin embedded tumors with mAb TM311.

Tissue ID	Staining Pattern	Category
91-7511	invasive tumor cells (TC) negative	N
92-1430	invasive TC 80% - 3+/4+; 20% - 0/1+	P
92-1935	" " 3+/4+	P
92-2534	" " 3+/4+	P
92-5156	" " 80% 3+/4+, 20% 1+/2+	P
92-5526	" " " 0/1+, 20% 2+	R
93-6216	" " 3+/4+	P
94-1287	" " >90% negative	N
94-3138	" " negative	N
94-8042	" " negative	N
	Totals:	50% positive 50% Reduced or Negative

Sections were stained as described in the text. Scoring of sections was as follows: tumors in which it was estimated that >60% of invasive cells were negative or showed reduced staining intensity (2+ or less on a scale of 1+ to 4+) were scored as negative. All others were scored as positive. Scoring was done independently by at least two investigators and only sections in which there was total agreement between investigators were included.

Figure Legends:

Fig. 1. Northern blot analysis of TM isoform expression in normal and cancerous human breast cells. A, TM RNA expression in the *in vitro* model cell lines. Lane assignments: 1)HMEC, 2)HBL100, 3)MCF10A, 4)184A1N4, 5)184A1N4TH, 6)SKBR3, 7)MDA-MB-231, 8)MCF7, 9)T47D. B, TM1 RNA expression in MDA-MB-231 cells treated with 5aza. Lane 1)Control HMEC, 2)untreated MDA-MB-231, 3)5aza-treated MDA-MB-231. C, TM1 RNA in MDA-MB-231 cells treated with TSA or TSA plus 5aza. Lane assignments: Control 184A1N4 (1), MDA-MB-231 cells either untreated (2), treated with TSA for 6h (3), 12h (4), 24h (5) and 36h (6) or treated with 5aza plus 6h (7), 12h (8), 24h (9) or 36h (10) TSA or 5aza alone (11).

Fig. 2. Western blot analysis of TM protein expression in the *in vitro* model cell lines. A, mAb TM311. Lane assignments: 1)HMEC, 2)MCF10A, 3)184A1N4, 4)184A1N4TH, 5)SKBR3, 6)MDA-MB-231, 7)MCF7, 8)T47D, 9)MDA-MB-435. B, mAb CG1. Lane assignments: 1)3T3-L1, 2)HMEC, 3)184A1N4, 4)MCF10A, 5)SKBR3, 6)MDA-MB-231, 7)MCF7, 8)T47D, 9)MDA-MB-435. C, mAb LC24. Lane assignments: same as for mAb CG1 except that lane 3 has HBL100 instead of 184A1N4. The positions of molecular weight standards (arrows) are given on the left.

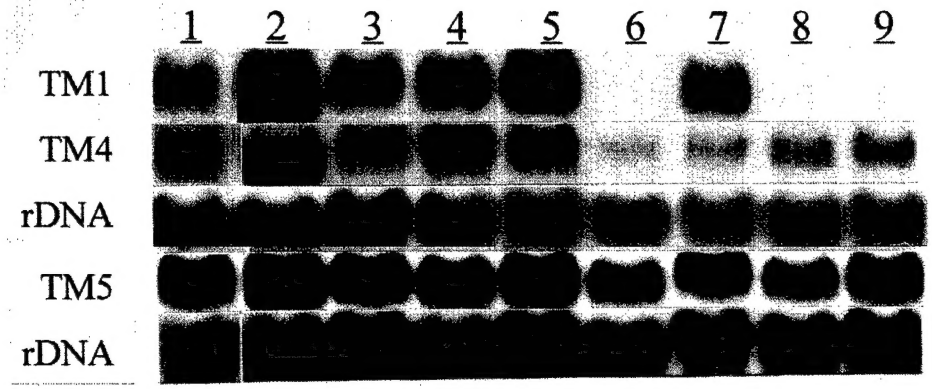
Fig. 3. Western blot analysis of TM1 expression in MDA-MB-231 cells after treatment with TSA or 5aza plus TSA. TM proteins were detected with mAb TM311. A, TSA-treatment. Lane assignments: Control HMEC (1), untreated MDA-MB-231 (2), MDA-MB-231 treated with TSA for 12h (3), 24h (4) or 36h (5). B, TSA plus 5aza treatment. Lane assignments: Control HMEC (1), MDA-MB-231 cells either untreated (2), 5aza-treated (3) or treated with 5aza plus TSA for

12h (4), 24h (5) and 36h (6). As suggested by results in Fig. 2, the TM311 bands are referred to as TM1 39kD and TM1 36kD isoforms. Actin signals are shown below.

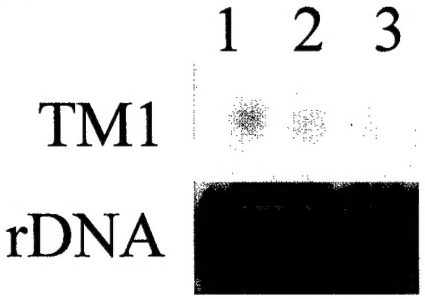
Fig. 4. Immunohistochemistry analysis of paraffin embedded tumors using mAb TM311. Panel A)Normal breast, panel B)Positive tumor, panels C and D, negative tumors. Sections were stained as described in the text. Slides were examined and photographed on an Olympus Model BH-2 microscope. Magnification in each panel is 200X.

Fig. 1

A.


1 2 3 4 5 6 7 8 9
TM1
TM4
rDNA
TM5
rDNA

B.


1 2 3
TM1
rDNA

C.

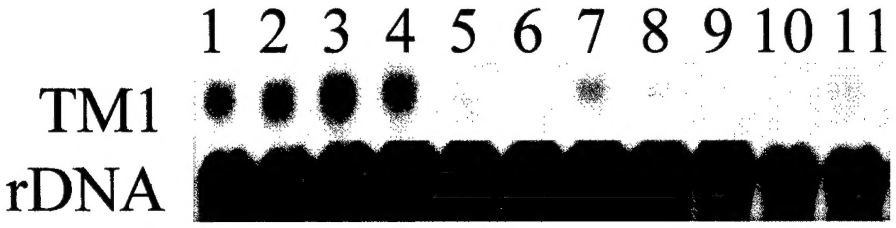
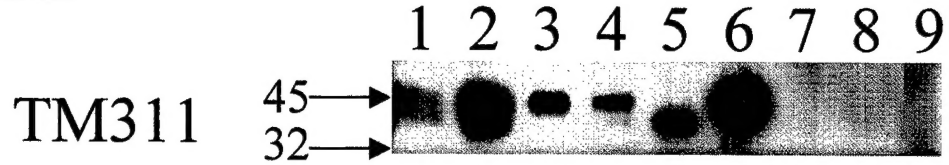

1 2 3 4 5 6 7 8 9 10 11
TM1
rDNA

Fig. 2

A.



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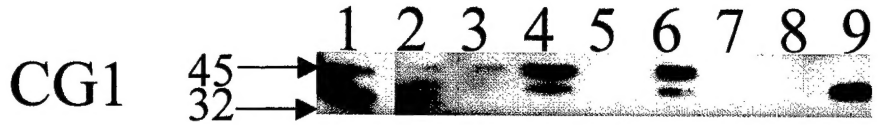


C.

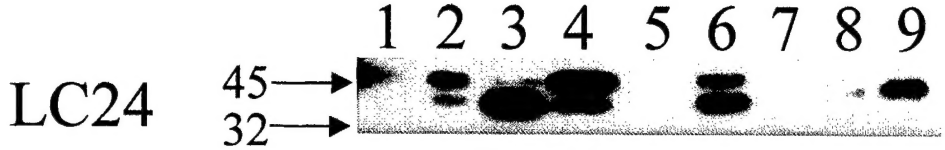
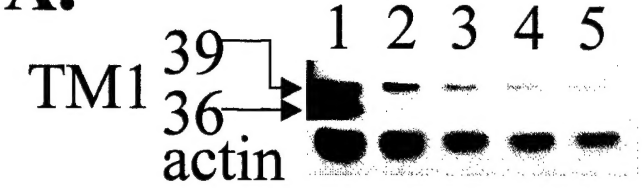


Fig. 3

A.



B.

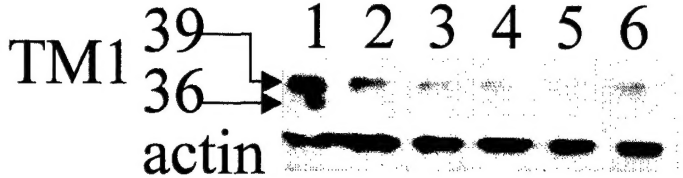


Fig. 4